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EXHIBIT 2

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Identification of *Mycobacterium marinum* virulence genes using signature-tagged mutagenesis and the goldfish model of mycobacterial pathogenesis

Kristin M. Ruley^{a,1}, John H. Ansele^{a,2}, Christopher L. Pritchett^a,
Adel M. Talaat^{a,3}, Renate Reimschuessel^b, Michele Trucksis^{a,c,*}

^a Center for Vaccine Development, University of Maryland School of Medicine, Baltimore, MD 21201, USA

^b Center for Veterinary Medicine, Food and Drug Administration, Laurel, MD 20857, USA

^c Medical Service, Veterans' Affairs Medical Center, University of Maryland School of Medicine, Baltimore, MD 21201, USA

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Abstract

Mycobacterium marinum, a causative agent of fish tuberculosis, is one of the most closely related *Mycobacterium* species (outside the *M. tuberculosis* complex) to *M. tuberculosis*, the etiologic agent of human tuberculosis. Signature-tagged mutagenesis was used to identify genes of *M. marinum* required for in vivo survival in a goldfish model of mycobacterial pathogenesis. Screening the first 1008 *M. marinum* mutants led to the identification of 40 putative virulence mutants. DNA sequence analysis of these 40 mutants identified transposon insertions in 35 unique loci. Twenty-eight out of 33 (85%) loci encoding putative virulence genes have homologous genes in *M. tuberculosis*.

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Keywords: *Mycobacterium marinum*; Signature-tagged mutagenesis; Mycobacterium; Virulence; Pathogenesis; Goldfish model

1. Introduction

Mycobacterium marinum is the causative agent of fish tuberculosis, infecting more than 150 species of salt- and fresh-water fish [1]. The organism, first isolated in 1926 from a salt-water fish [2], causes a systemic infection that ultimately leads to the death of the animal. Based on 16S rRNA homology and DNA–DNA hybridization studies [3,4], *M. marinum* has been recognized as one of the two most closely related *Mycobacterium* species, out-

side the *M. tuberculosis* complex, to *M. tuberculosis*. Both *M. tuberculosis* and *M. marinum* are intracellular pathogens that proliferate within macrophages in a non-acidic phagosome that does not fuse with the lysosome [5,6]. Although *M. tuberculosis* was identified as the causative agent of human tuberculosis over a century ago, progress towards identification of virulence genes in *M. tuberculosis* has been slow because of the difficulties associated with studying this organism.

Our laboratory has used *M. marinum* and the goldfish model (*Carassius auratus*) [7] to study mycobacterial pathogenesis. *M. marinum* is a natural pathogen of the goldfish, and thus this model is a natural infection model. We report here that we have coupled *M. marinum* and the goldfish model with signature-tagged mutagenesis (STM) [8] for the large-scale screening of *M. marinum* mutants to identify those with attenuated phenotypes. In addition, we have taken the putative *M. marinum* virulence genes and have identified their *M. tuberculosis* virulence gene homologues.

* Corresponding author. Present address: Division of Infectious Diseases and Immunology, University of Massachusetts Medical School, Worcester, MA 01605, USA. Tel.: +1 (508) 856-3410; Fax: +1 (508) 856-5463.

E-mail address: michele.trucksis@umassmed.edu (M. Trucksis).

¹ Present address: Center for Human Virology and Biodefense, Thomas Jefferson Medical College, Philadelphia, PA 19107, USA.

² Present address: Qualyst, Inc., P.O. Box 30189, Raleigh, NC 27622-0189, USA.

³ Present address: Animal Health and Biomedical Sciences, School of Veterinary Medicine, University of Wisconsin, Madison, WI 53706, USA.

2. Materials and methods

2.1. Fish

Goldfish (*C. auratus*), weighing 25–30 g, were obtained from Hunting Creek Fisheries, Thurmont, MD, USA. Fish were housed in a quarantine area at the Food and Drug Administration's Center for Veterinary Medicine, Laurel, MD, USA.

2.2. Bacterial cultures and growth

M. marinum strain ATCC 927 (fish isolate) was obtained from the American Type Culture Collection (Rockville, MD, USA). Bacteria were grown as described previously [7]. Mutant strains were grown with the addition of 50 µg ml⁻¹ kanamycin (Sigma, St. Louis, MO, USA).

2.3. Construction of the signature-tagged shuttle vector

The mycobacterial shuttle vector pYUB285 was a gift from William Jacobs Jr., Albert Einstein College of Medicine, Bronx, NY, USA [9]. A *HincII* fragment from pSTM14 (obtained from Christoph Tang, Oxford University, Oxford, UK) containing an *aph* cassette was exchanged for the *aph* gene of IS1096 to provide a unique *Bgl*III site, creating pAT20. The DNA signature tag sequences synthesized as described below were inserted into the *Bgl*III site in pAT20 to generate uniquely tagged plasmids.

2.4. Cloning and selection of the unique DNA tags

The 90-bp signature tags were polymerase chain reaction (PCR)-amplified from the RT1 oligonucleotide template [8] using primers P5 (5'-CTAGGTACCTACAACCTC-3') and P3 (5'-CATGGTACCCATTCTAAC-3') that contained *Bam*HI restriction sites at the 5' ends. The amplified tags were digested with *Bam*HI (Invitrogen, Carlsbad, CA, USA), gel-purified (Qiaex II, Qiagen, Valencia, CA, USA) and ligated to the *Bgl*III-digested, dephosphorylated pAT20 plasmid. The resulting plasmids were transformed into *Escherichia coli*, plasmids isolated, and screened for the ability to amplify their PCR tags and for lack of cross-hybridization against other plasmids. Fifty-three plasmids were designated the master plasmid collection. *M. marinum* ATCC 927 strain was grown and prepared for electroporation as previously described [10]. Each plasmid from the master collection was individually electroporated into *M. marinum*.

2.5. Screening of tagged mutant library

M. marinum mutants were grown in individual wells of a microtiter plate. Growth was monitored daily with an ELISA plate reader and mutants with significantly re-

duced growth rates in vitro were excluded from input pools. When OD₅₉₅ reached 0.8, mutants were pooled into a single inoculum and sonicated for 3 min at power level 3, while cooling using a cup horn accessory attached to a cell disrupter (model W-220F, Heat Systems, Ultrasonics, Farmingdale, NY, USA). Each pool consisted of 48–53 uniquely tagged mutants.

A portion of the pool was used as a template for PCR amplification of the signature tags, which were then radiolabeled to generate the input PCR probe for hybridization to the master plasmid collection membrane, using the following protocol. The input pool was boiled and supernatant served as template for the PCR amplification of the tags. The PCR reaction contained 5 µl of template, 60 µM primers P2 (5'-TACCTACAACCTCAAGCT-3') and P4 (5'-TACCCATTCTAACCAAGC-3') [8], 200 µM dNTPs, PCR buffer, 2.2 mM MgCl₂, and 1 U *Taq* DNA polymerase (Invitrogen). The amplification cycle was 5 min at 96°C followed by 30 cycles of 95°C for 1 min, 45°C for 30 s, and 72°C for 10 s. The 90-bp product of the initial PCR reaction was extracted using a Qiaex II column (Qiagen). The purified PCR fragments were radiolabeled with [α -³²P]dCTP (Amersham Pharmacia Biotech, Piscataway, NY, USA) using the following: 0.5 µl of template (the 90-bp product), 5 µl [α -³²P]dCTP (500 µCi), 60 µM primers P2 and P4, 200 µM dATP, dGTP, dTTP, PCR buffer, 2.2 mM MgCl₂, and 1 U *Taq* polymerase. The amplification protocol was as above. The radiolabeled tags were digested with *Hind*III (Invitrogen) (to release the invariable region of the tags) and used to probe the dot blot membrane of the master plasmid collection as described below.

The remaining portion of the input pool of mutants was adjusted to 2.0 × 10⁷ CFU ml⁻¹. Each pool of mutants (0.5 ml) was inoculated intraperitoneally into three goldfish [7]. The number of CFU ml⁻¹ in each inoculum was determined by plating serial dilutions on 7H10 agar.

Fish were killed 1 week post inoculation and livers harvested, homogenized and plated. Colonies were scraped from the plates and suspended in phosphate-buffered saline (PBS). This suspension, the output pool of mutants, was sonicated, boiled, centrifuged, amplified and labeled as described above for the input pool.

2.6. Hybridization

A dot blot membrane representing the master plasmid collection was generated using a Bio-dot® apparatus (Bio-Rad, Hercules, CA, USA) and a Hybond®-N (Amersham Pharmacia Biotech) nylon membrane as per the manufacturer's instructions. The membrane was hybridized, washed and autoradiographed with the pool-specific radiolabeled PCR product (input or output pool) using standard protocols (Bio-Rad). The membranes were stripped of the input pool probe and used for hybridization with the corresponding output pool probe. An arbitrary scoring method (0–4; 0, no signal, 4, most intense signal) was used

to analyze the autoradiographs by two independent, blinded readers. Only mutants that showed a decrease in signal of >2 points (input–output) in both animals by both readers were considered attenuated by this screen.

2.7. Competitive index (CI) assay

The wild-type *M. marinum* ATTC 927 and mutant strains were grown as previously described [7], harvested, resuspended in sterile PBS to achieve an inoculum of 2×10^7 CFU ml $^{-1}$, and sonicated as above. Equal quantities of the wild-type and mutant inocula were combined and 0.5 ml used to inoculate three to six fish intraperitoneally for each assay. The number of CFU of wild-type and mutant strain in the mixed inoculum was determined by plating on 7H10 agar with and without kanamycin (mutant). Fish were killed after 1 week and the livers harvested, homogenized and plated as above. The CI was calculated using the formula: $[(\text{CFU mutant}_{\text{output}})/(\text{CFU wild-type}_{\text{output}})]/[(\text{CFU mutant}_{\text{input}})/(\text{CFU wild-type}_{\text{input}})]$ [11].

2.8. Identification of transposon and flanking DNA sequences by cosmid cloning of the STM mutants

Chromosomal DNA from each mutant was prepared as described by Belisle and Sonnenberg [12], partially digested with *Sau*3AI (Invitrogen) and 30-kb fragments li-

gated to the cosmid vector pHC79 [13] previously digested with *Bam*HI and dephosphorylated with shrimp alkaline phosphatase. The ligation was packaged using the Giga-pack[®] III XL Packaging Extract according to the manufacturer's instructions (Stratagene, La Jolla, CA, USA). *E. coli* strain VCS257 (Stratagene) was transformed with packaged phage, and clones containing the transposon with the flanking DNA were selected on Luria agar with kanamycin. Cosmids were sequenced using primers T340 (5'-GCTCTTCCTCTTGCTCTTCC-3') and T343 (5'-TCCATCATCGGAAGACCTCG-3') which were designed to sequence at either end of the transposon moving outward into the flanking *M. marinum* DNA sequence. The open reading frame (ORF) interrupted by the transposon in the *M. marinum* mutant was identified and compared to the *M. tuberculosis* genome using BLAST search. The ORFs interrupted in *M. marinum* mutants 67.1, 88.2, 125.20, 129.8, and 68.6 have been deposited in the GenBank database under accession numbers AY117693, AY121836, AY121837, AY517550, and AY517551 respectively.

2.9. Statistical analysis

CI values are expressed as means \pm S.E.M. Means were compared by two-tailed Student's *t*-test for unpaired data. Differences were considered significant if $P \leq 0.05$.

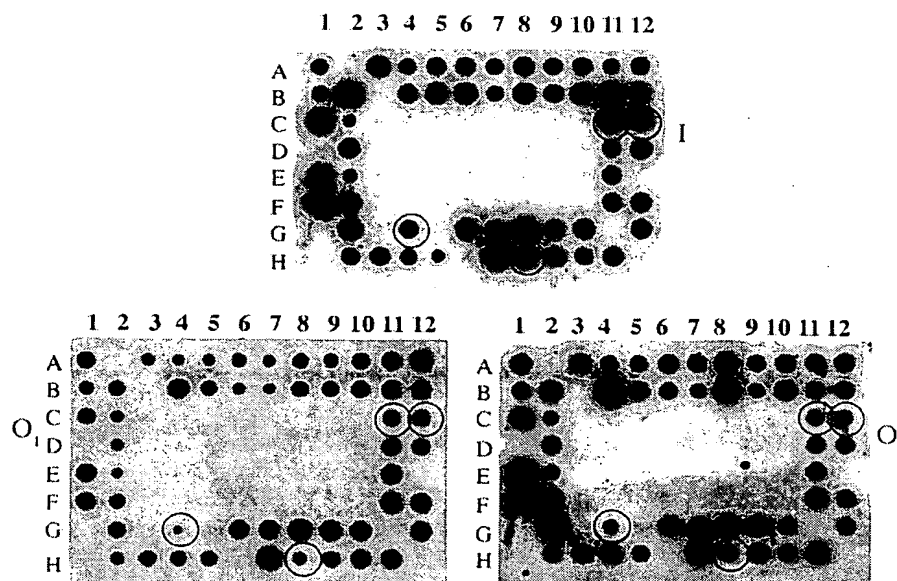


Fig. 1. Identification of *M. marinum* genes attenuated in virulence. An example of a single pool's hybridization pattern is shown. Hybridization of tags amplified and labeled from a specific pool of mutants in the inoculum (input probe, I), and with tags amplified and labeled from the corresponding output pool from bacteria that were recovered from two different goldfish livers (output, O₁ and O₂) to the master plasmid collection dot blot. Plasmids C11, C12, G4, and H8 (circled) showed a decrease of at least 2 units in hybridization signal between the input probe (I) and both output probes (O₁ and O₂).

3. Results and discussion

3.1. Screening of *M. marinum* tagged transposon mutant library for virulence genes

A total of 1008 mutants (in 20 pools) were screened in the goldfish. Two infection periods (1 and 4 weeks) were examined with the first pool. There was no difference in the mutants identified at either time in the first pool, so even though additional mutants may have been identified at the later time point, an infection period of 1 week was used in subsequent pools. Because the highest colony count was obtained from the liver, this organ was used to harvest the output pool. Mutants were identified as attenuated if they showed a reproducible decrease of at least 2 units in hybridization signal between input and output pools in two animals (Fig. 1). Forty putative virulence mutants were identified in the STM screen of 1008 mutants (4.0%).

Table 1

Comparison of the proteins encoded by the STM-identified genes of *M. marinum* to the *M. tuberculosis* proteome

Functional class	<i>M. marinum</i> mutant	<i>M. tuberculosis</i> homologue ^a	Identity to <i>M. tuberculosis</i> (%)	<i>e</i> value ^b
PPE/PE	76.1	Rv1705c, PPE	67	2.00 e ⁻⁵¹
	32.2	Rv0159c, PE	62	1.00 e ⁻⁵²
	58.14	Rv2356c, PPE	62	3.00 e ⁻⁴⁵
	42.2	Rv0305c, PPE	60	1.00 e ⁻⁷²
	60.2	Rv3347c, PPE	60	4.00 e ⁻²¹
	80.8	Rv1548c, PPE	57	5.00 e ^{-10.13}
	61.5	Rv3159c, PPE	38	1.00 e ^{-20.11}
Cell wall-related	114.4	Rv0644c, methoxy mycolic acid synthase, <i>mmaA2</i>	86	3.00 e ⁻⁸⁴
	49.7	Rv1428c, acyl transferase	70	4.00 e ⁻³¹
	97.4	Rv2831, enoyl-CoA hydratase/isomerase, <i>echA16</i>	43	5.00 e ⁻⁰⁸
Metabolism	72.10	Rv3272, L-carnitine dehydratase	84	9.00 e ⁻⁴⁵
	39.14	Rv2013c, methyl transferase	77	3.00 e ⁻⁴⁵
PKS	62.2	Rv0101, non-ribosomal peptide synthetase module, <i>nrp</i>	51	2.00 e ⁻³¹
	49.6	Rv1013, polyketide synthase, <i>pkS16</i>	46	3.00 e ⁻¹⁹
	86.1	Rv2048c, polyketide synthase, <i>pkS12</i>	39	2.00 e ⁻⁰⁸
	95.3	Rv2933, polyketide synthase, <i>ppsC</i>	34	5.00 e ⁻²²
Amino acid/nucleic acid/synthesis	39.2	Rv3137, <i>cysQ</i>	42	4.00 e ⁻⁰⁶
	27.1	Rv1285, <i>cysD</i>	22	0.54
	80.1	Rv1981c, ribonucleotide reductase, <i>rdpF</i>	71	3.00 e ⁻⁷⁷
Unknown	18.5	Rv3234c, unknown	85	2.00 e ⁻³⁸
	102.4/114.7	Rv2348c, unknown	82	3.00 e ⁻⁴¹
	1.4	Rv3901c, unknown	65	9.20 e ⁻⁰¹
	95.18	Rv0236, unknown	64	2.00 e ⁻⁵³
	38.3	Rv0455c, unknown	62	4.00 e ⁻³⁴
Miscellaneous	58.15/62.6	Rv1984c, cutinase	48	2.00 e ⁻¹⁹
	68.12	Rv0797, transposase	29	7.00 e ⁻²²
	62.20	Rv0449c, FAD binding protein	76	6.00 e ⁻⁴²
	41.2	Rv0822c, regulator with AraC signature	73	2.00 e ^{-22.13}
No <i>M. tuberculosis</i> homologue	67.1	AY117693 ^c		
	88.2	AY121836 ^c		
	125.20	AY121837 ^c		
	129.8	AY517550 ^c		
	68.6	AY517551 ^c		

^aDetermined by comparison of sequences using BLASTX network services; proposed protein function as reported by *M. tuberculosis* genome annotation [14].

^bBLASTX *e* value.

^cGenBank database nucleotide sequence accession number.

3.2. Sequence analysis of the attenuated virulence mutants

The chromosomal DNA from each mutant was cosmid-cloned and plasmids from kanamycin-resistant clones (containing the transposon with the flanking *M. marinum* DNA) were sequenced as described in Section 2. The nucleotide sequences of the flanking regions (400–600 bp per flanking region) were determined and analyzed by BLASTX network services for identical or similar proteins in the SwissProt database (Table 1).

Sequence was obtained for 37 of the 40 mutants identified in the STM screens. One mutant had two transposon insertion sites, as identified by Southern analysis (data not shown), and was eliminated from further analysis and two mutants were unable to be sequenced. Of the remaining 37 mutants, two pairs of mutants (58.15/62.6 and 102.4/114.7) had insertions into the same gene, although the insertion site was different in each case. Thus, our screen identified 35 unique loci in the 40 mutants identified by STM.

Twenty-eight of the *M. marinum* mutants (85%) which have the transposon inserted into a putative virulence gene encode a protein homologous to a protein encoded by the *M. tuberculosis* genome. One mutant (67.1) is most homologous to a sulfate adenylyltransferase in *Pyrococcus abyssi*, another (125.20) to a *Streptomyces* ORF, and three mutants (88.2, 129.8, and 68.6) have no known homologues.

3.3. *M. marinum* mutants classified by functional role

Mutants identified by STM were grouped into six classes based on the functional role of the protein encoded by the disrupted gene (Table 1). One class of mutants belongs to the PPE/PE family of genes and encodes a signature Pro-Glu (PE) amino acid sequence [14]. Ramakrishnan et al. [15] have identified some members of this gene family as being selectively expressed in macrophages. Although the function of these genes is not known, they are theorized to be involved in replication in macrophages, persistence in granulomas, and antigenic diversity [15,16]. A second class of genes is involved in cell wall-related functions. Mutant 114.4 is disrupted in a methoxymycolic acid synthase gene (*mmaA*). In pathogenic mycobacteria, *mmaA* genes are believed to catalyze cyclopropanation of fatty acids, which possibly protects the fatty acid chain from degradation by oxygen free radicals generated within macrophages [17]. Mutant 97.4 has a disruption in a gene homologous to an enoyl-CoA hydratase, involved in elongation of the fatty acid chains in mycobacteria [18].

Genes involved in metabolism make up the third cate-

gory of mutants. A mutant in this class (72.10) has a disruption in a L-carnitine dehydratase homologue, which is involved in anaerobic metabolism of L-carnitine as a nitrogen and/or carbon source [7262]. Other genes disrupted in mutants from this class are homologous to those found in signal transduction pathways, including a methyl transferase.

A fourth group of mutants (62.2, 86.1, 95.3, 49.6) belongs to the polyketide synthase (PKS) family of genes. *pks* genes are found throughout the *M. tuberculosis* genome and are believed to be involved in the synthesis of cell wall lipids [19].

A fifth class of mutants is involved in amino acid or nucleic acid synthesis. Two mutants are disrupted in genes homologous to *cysD* (27.1) and *cysQ* (39.2), in the *cys* operon, which are required for cysteine biosynthesis, suggesting that cysteine may be of limited availability in the macrophage.

The final group of mutants has disruptions in genes with no known function. These five mutants are highly homologous (62–85%) to hypothetical proteins in *M. tuberculosis*.

Four genes (mutants 58.15/62.6, 68.12, 62.20, 41.2) identified by STM that could not be grouped according to a collective function were classified as miscellaneous.

3.4. Competition assays of mutants identified by STM

The CI assay was used to further establish that the mutants identified by STM were attenuated in virulence. In the assay, mixed infections with mutant and wild-type

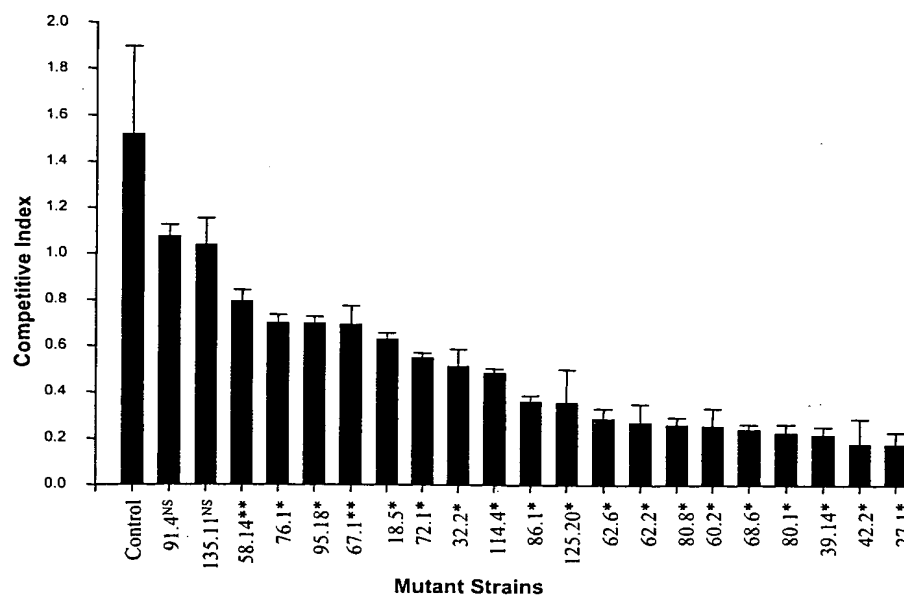


Fig. 2. CI assay of selected mutants identified by STM. The x-axis shows mutant strains co-infected with wild-type strain ATCC 927. Mutant 32.1 was not identified as attenuated by STM and was used as a non-attenuated control strain. The y-axis represents CI values calculated using the formula: $[(\text{CFU mutant}_{\text{output}})/(\text{CFU wild-type}_{\text{output}})]/[(\text{CFU mutant}_{\text{input}})/(\text{CFU wild-type}_{\text{input}})]$ [11]. The results are given as mean \pm S.E.M. for three to six fish per co-infection. * $P < 0.001$, ** $P = 0.002$, NS: not significant, $P > 0.05$.

strains are used to provide an in vivo measure of virulence attenuation referred to as the CI. Non-attenuated mutants have a CI=1.0, whereas attenuated mutants have a CI<1.0, with the most attenuated mutants having the lowest CI values. Of the 21 mutants tested, 19 mutants (90%) were attenuated in competition studies, with CI values ranging from 0.18 to 0.78 (Fig. 2).

3.5. *M. marinum* and the goldfish as a model system to identify genes involved in mycobacterial pathogenesis

There has been much speculation about the identity of virulence factors of *M. tuberculosis*, however few have been confirmed using Koch's molecular postulates. The search for virulence genes has benefited from molecular biological methods such as STM. However, the animal model chosen for use with STM is vital to the success of the screen. For this reason, when possible, a natural infection model is preferable as it may discern factors involved in the establishment and persistence of infection, as well as those factors involved in subversion and resistance to host defenses.

The purpose of this study was to assess the ability of the goldfish model of mycobacterial pathogenesis coupled with STM to identify virulence genes of *M. marinum*. Through examination of the predicted protein sequence of the *M. marinum* virulence genes, we would then identify bacterial virulence factors operative in disease caused by *M. tuberculosis*. We predicted that the genetic and pathogenic relatedness of these two *Mycobacterium* species as established by several investigators [3,4] would allow us to utilize our *M. marinum* natural infection model to discover virulence genes of *M. tuberculosis*. These genes would serve as mutation targets for construction of live-attenuated vaccines, as new drug targets, and to increase our understanding of the mechanisms by which pathogens cause disease.

Our results demonstrate the utility of this approach as we have identified 33 putative virulence genes of *M. marinum* and found that all but five have homologues in *M. tuberculosis*. Of note, our results identified some of the same genes/gene classes identified in an STM screen performed with *M. tuberculosis* in the mouse model [20], validating the use of *M. marinum* as a surrogate for *M. tuberculosis*. These genes include *pks* genes, genes belonging to the PPE family of genes, and a transcriptional regulator with an AraC signature.

In comparing our results with those of Chan et al. [21], who looked for *M. marinum* genes specifically expressed in the frog granuloma, we identified no common genes. There are several reasons why the screens were not congruent. First, neither screen was saturating. Second, different reporter methods were used in the two screens; the STM screen measures a mutant's ability to survive in vivo, while the Chan et al. study investigated those genes whose promoters were specifically expressed in the granu-

loma. Thus, Chan et al.'s study was not meant to identify virulence genes but to examine *M. marinum* gene expression in granulomas.

The limitations of the application of STM to our goldfish model of mycobacterial pathogenesis include the common disadvantages of STM, in that the screen will be unlikely to detect mutations in virulence determinants whose functions can be transcomplemented by the presence of other strains, i.e. toxins. Further, genes that are essential or those whose virulence functions are redundant are unlikely to be identified. In addition, polar effects on downstream genes can complicate the analysis of transposon mutants. This is most likely to occur when the transposon is located in a potential operon. But since most operons contain groups of functionally regulated genes, these insertions will remain informative. Additionally, two mutants identified by STM were not attenuated in the CI assay (Fig. 2). These two mutants represent false-positive mutants, which have also been seen in other STM screens [20]. Finally, our model bypasses the early stages of the infection process because of the intraperitoneal route of infection. Thus, bacterial factors important in adherence and colonization of surfaces will not be identified.

It is evident from the size of the *Mycobacterium* genome (> 4 Mb) [14] that our screen was not comprehensive, as a complete screen would require more than 4000 transposon mutants. The finding that only two of the genes were identified twice supports this conclusion. In addition, although the transposon in our system appeared to insert randomly, all transposons exhibit some sequence bias for transposition. The identification of mutants with insertions in genes encoding previously identified virulence determinants validates the approach used in the study. Finally, the identification of genes not previously known to be involved in virulence provides new insights into the basis of mycobacterial pathogenesis.

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